Improved preparation method for lysogangliosides

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Summary Lyso-GM_3 and -GM_1 gangliosides were prepared from the corresponding N,N'-dideacylated gangliosides using N-trifluoroacetylation at the sphingosine moiety, followed by N-acetylation and mild saponification. The blocking reaction was performed using a water–ether bilayer system at alkaline medium, in which the N-trifluoroacetylation occurred predominantly at the lipid moiety. Through the procedure, lysoGM$_3$ and lysoGM$_1$ were obtained with higher yields from the corresponding dideacylated gangliosides than through the previous method using 9-fluorenylmethoxycarbonyl chloride as a blocking group or of direct N-acetylation of it on liposomes containing starting ganglioside and other lipid. Chemical structures of the lysogangliosides and the synthetic intermediates were confirmed by the proton nuclear magnetic resonance spectrometry and negative fast atom bombardment–mass spectrometry.—**Gasa, S., K. Kamio, and A. Makita.** Improved preparation method for lysogangliosides. *J. Lipid Res.* 1992. **33:** 1079–1084.

Supplementary key words lysoGM$_3$ • lysoGM$_1$ • trifluoroacetylation • S-ethyltrifluorothioacetate

Lysogangliosides are reported to modulate cellular signaling (1). To study the signaling mechanism by lysogangliosides and to utilize them as ligands for ganglioside-binding proteins such as synthases, it is necessary to prepare lysogangliosides from native gangliosides, because of their undetectable content in many tissues. The lysolipids are also very useful for making labeled lipids.

The chemical preparation of lysoglycosphingolipid is ordinarily performed by saponification (2) or hydrazinolysis (3) of the native glycolipid followed by isolation of the lysocompounds by chromatography. These procedures have been applied successfully to glycolipids which do not contain sialic acid and/or N-acetylhexosamine. However, chemical deacylation of glycolipids containing sialic acid and/or N-acetylhexosamine produces, in general, a variety of totally and partially deacylated derivatives in addition to lysoglycolipids, the amounts of which are not high because of lack of selective deacylation at the ceramide moiety. Thus, the direct, one-step deacylation at the lipid moiety is not suitable to produce lysoglycolipids of the complex sugar chain type. Free amino groups of neuraminic acid and/or hexosamine in a deacylated ganglioside which also has N-free lipid moiety was able to be N-acylated by keeping lipid-amine group free in a liposome environment (4). Alternatively, the amino group at the lipid moiety of the deacylated gangliosides was first blocked using (9-fluorenylmethoxy)carbonyl chloride (Fmoc-Cl) in a water–organic solvent bilayer system followed by N-acetylation of the sugar; deblocking generated a lysoganglioside with higher yield (5).

S-ethyl trifluorothioacetate (ETFTA) was previously used as a blocking agent for amino residues on 6-aminohexyl phosphate to make the N-trifluoroacetyl derivative (6). The trifluoroacetylation reaction was mild and slow under basic conditions at pH 8.0, and the trifluoroacetyl group was removable from the N-trifluoroacetyl derivative in a mild alkaline solution at pH 11; no degradations occurred in the N-acetyl group or at the glycosidic linkage in the ganglioside structure. Thus, in this report, we used the N-trifluoroacetylation of dideacylated monosialoganglioside for the preparation of the corresponding lysoganglioside.

MATERIALS AND METHODS

Chemicals

ETFTA was purchased from Aldrich Chemical Company (Milwaukee, WI). Sep-Pak C_{18} cartridges (1-ml bed) were obtained from Waters (Milford, MA). Precoated thin-layer chromatography (TLC) plates (Silica Gel 60) were from Merck (Darmstadt, Germany). Other reagents were of analytical grade. Gangliosides GM_3 containing NeuGc and GM_1 were prepared from equine erythrocytes (7) and porcine brain, respectively.

Preparation of lysogangliosides

Deacylation of ganglioside. The ratio of the solvent mixture is expressed by volume. Ganglioside, GM_3 or GM_1 (20–50 μmol) was N-deacylated in a solution (10 ml) of 1 M methanolic potassium hydroxide according to the method of Neuenhofer et al. (5), with slight modification. The course of the reaction was monitored on TLC plates developed in chloroform–methanol–water–acetic acid 50:50:9:1 (solvent A). After the deacylation was completed (16 to 20 h), the mixture was neutralized with acetic acid, evaporated, and desalted by Sep-Pak cartridge (8). The Sep-Pak equipped with a glass syringe used one cartridge per 7 μmol of starting ganglioside. The desalting was performed with 10 ml each of methanol–water 3:7 and water, and the product was eluted with 2 ml of ethanol and 10 ml

Abbreviations: Nomenclature and abbreviations for gangliosides are used as recommended by Svennerholm (1972. *Methods Carbohydr. Chem.* **4:** 464–474); in N,N'-dideacylGM_3 or -dideacylGM_1, N denotes amino-N at ceramide and N' at sialic acid; Sph, sphingosine; ETFTA, S-ethyl trifluorothioacetate; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; FAB–MS, fast atom bombardment–mass spectrometry; Fmoc-Cl, 9-fluorenylmethoxycarbonyl chloride.

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of chloroform–methanol–water 60:30:4.5 (9). The slowest-migrating ninhydrin-positive product was purified by silica gel column chromatography (1 × 20 cm) eluted with 10 bed-vol of chloroform–methanol–water by raising the polarity of the solvent mixture, or by preparative TLC developed with solvent A. The column chromatography was started with the mixture composition of 70:30:3 and the ratios of methanol and water were increased stepwise. The desired product was eluted by ratios of 20:80:8 to 0:100:10 chloroform–methanol–water.

N-Trifluoroacetylation of dideacylganglioside. The purified N,N'-dideacylated GM_3 or GM_1 was suspended in 3 ml of aqueous 0.5 M sodium bicarbonate and 2 ml of ether in a screw-capped glass tube (1 × 5 cm). Small portions (20 μl) of ETFTA were added to the mixture under vigorous stirring at room temperature after a 1-h interval. The reaction was followed by TLC with solvent A, and the addition of ETFTA was continued until the amount of ninhydrin-positive compound became minimum (10–18 h).

N-Reacetylation of the N-trifluoroacetylated compound. After the mixture was adjusted to pH 8 with 1 M sodium bicarbonate, the organic solvent was evaporated. The aqueous remainder was N-acetylated by the addition of small portions (10 μl) of acetic anhydride until pH 5 was attained (2–5 h).

Removal of trifluoroacetyl group and purification of lysoganglioside. The N-acetylation mixture was neutralized with 1 M sodium bicarbonate, desalted by Sep-Pak cartridge, evaporated to dryness, and chromatographed on a silica gel column as described above. The N-trifluoroacetyl N'-acetyl dideacyl GM_3 or -GM_1 was dissolved in 2 ml of chloroform–methanol–water 30:60:8 followed by the addition of concentrated methanolic sodium methoxide at pH 11. After 2 h or more at room temperature, the mixture was neutralized with acetic acid and desalted. The crude lysoganglioside obtained was further purified by silica gel column chromatography. The lysoganglioside was eluted with chloroform–methanol–water (50:50:5 for lysoGM$_3$ and 30:70:7 for lysoGM$_1$). Before proceeding to the next steps described above, the intermediates were isolated from the reaction mixture in order to confirm the chemical structure by proton nuclear magnetic resonance (NMR) and negative fast atom bombardment–mass spectrometry (FAB–MS).

NMR measurement

Proton NMR spectra of purified lysogangliosides (approximately 1 mg), or the intermediates (1 mg) through the above reactions were obtained in 0.4 ml of dimethylsulfoxide-d6 containing 2% deuterium oxide at 90°C on a Varian JNM GX-500 spectrometer in a Fourier-transform mode, at the High Resolution NMR Laboratory of Hokkaido University as described previously (10). GM_3 and GM_1 were measured in the absence of deuterium oxide. The frequency and the sweep width were 500 MHz and 5 kHz, respectively. Chemical shifts were indicated by distance (ppm) from tetramethylsilane as an internal standard.

FAB–MS measurement

FAB–MS spectra were measured using a JEOL JMS-DX-300 mass spectrometer equipped with a JMA-DA-5000 Mass Data System at GC–MS and the NMR Laboratory of Faculty of Agriculture of Hokkaido University. The sample was bombarded by Xe gas with 6 kV (20 mA) in a matrix of glycerol, and the fragments were accelerated at 2 kV.

RESULTS

Preparation of lysogangliosides

The TLC patterns of the intermediates and final product in the preparation of lysoGM$_3$ and lysoGM$_1$ are presented in **Fig. 1**. Here, the dideacylated GM_3, the mono-trifluoroacetyl product, and the lysolipids were positively stained by ninhydrin reagent.

N-Deacylation at ceramide and sialic acid moieties of the gangliosides was completed within 20 h at 100°C in a methanolic 1 M potassium hydroxide solution as reported previously (5), giving N,N'-dideacylGM$_3$ with an yield of 80%. In the reaction, however, some by-products appeared faintly on TLC plates. For example, GM_3 gave free neuraminic acid, glucosylsphingosine, and lactosylsphingosine with respective yields of less than 3%. These degraded products from GM_3 were identified by

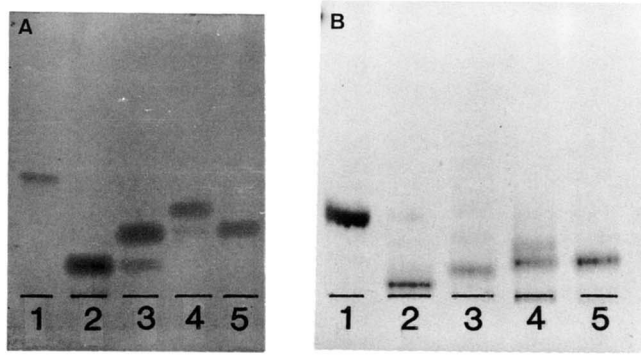


Fig. 1. TLC pattern of synthetic intermediates for the lysogangliosides. In panel A, lane 1 shows intact GM_3 containing N-acetyl neuraminic acid; lane 2, N,N'-dideacyl GM_3; lane 3, N-trifluoroacetyl N'-deacetyl dideacyl GM_3; lane 4, N-trifluoroacetyl N'-acetyl GM_3; lane 5, lysoGM$_3$. In B, lane 1 indicates intact GM_1; lane 2, N,N'-dideacylGM$_1$; lane 3, N-trifluoroacetyl N'-deacetyl GM_1; lane 4, N-trifluoroacetyl N'-acetyl dideacyl GM_1; lane 5, lysoGM$_1$. The trifluoroacetyl group was slightly released from the trifluoroacetyl derivatives of N,N'-dideacyl gangliosides during storage in $CHCl_3$–CH_3OH–H_2O solution, giving N,N'-dideacyl gangliosides (each lower band in lane 3) and N'-acetyl dideacyl gangliosides (each lower band in lane 4). The plate was developed with $CHCl_3$–CH_3OH–H_2O–CH_3COOH (60:40:8:1), and the spots were stained by orcinol–sulfuric acid reagent.

means of proton NMR spectra (data not shown). Similarly, alkaline hydrolysis of GM_1 produced a major deacylated compound which was characterized as N,N'-dideacylGM_1 by NMR and FAB-MS analyses (see below). Although the trifluoroacetylation reaction of the dideacylated GM_3 and GM_1 was slow as compared to the reaction with Fmoc-Cl (5), the yields of the N-trifluoroacetyl N'-deacyl-GM_3 and -GM_1 were 80% and 70%, respectively. Mobility of the N-trifluoroacetyl N'-deacyl GM_3 on TLC was similar to that of lysoGM_3 (Fig. 1A, lanes 3 and 5), whereas those derivatives of GM_1 were slightly different (Fig. 1B, lanes 3 and 5). In the reaction, an N,N'-di-trifluoroacetylated product was obtained with an yield of less than 10%, and was convertible to the starting N,N'-dideacyl ganglioside by mild alkaline hydrolysis. The reversely monotrifluoroacetylated compound on neuraminic acid moiety, N'-trifluoroacetyl N-deacyl derivative, was negligible in the reaction of both dideacylated gangliosides.

N-reacetylation of the N-trifluoroacetyl N'-deacetyl derivatives proceeded quantitatively. Both the N-trifluoroacetyl N'-acetyl gangliosides obtained migrated faster than the respective N-trifluoroacetyl N'-deacetyl derivatives (lanes 4 in Fig. 1A and 1B). The N-trifluoroacetyl group was completely removed under mild alkaline hydrolysis. The trifluoroacetyl residue was very labile, and was removed from the N-trifluoroacetyl derivatives during storage in chloroform–methanol–water 60:30:4.5 at room temperature for 2 weeks as shown in Fig. 1A, lanes 3 and 4, and Fig. 1B, lane 4. Throughout the preparation procedures described herein, lysoGM_3 and lysoGM_1 were obtained from the corresponding starting gangliosides with average yields of 65% in four different experiments and with 55% in three experiments, respectively.

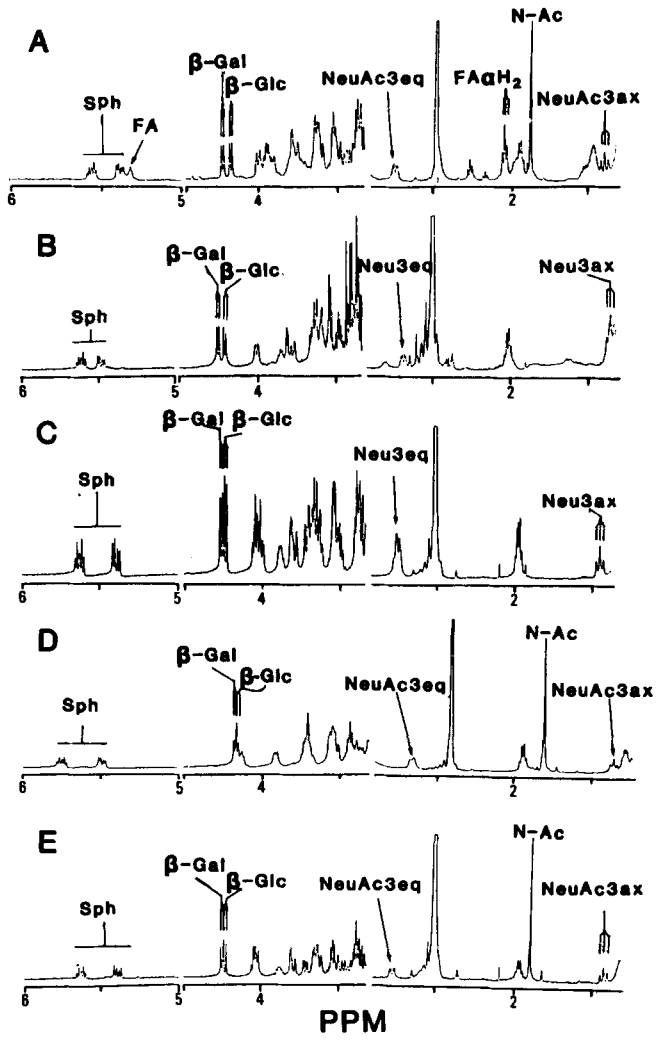


Fig. 2. NMR spectra of synthetic intermediates for the lysoGM_3. A, GM_3 containing N-acetyl neuraminic acid; B, N,N'-dideacyl GM_3; C, N-trifluoroacetyl N-deacetyl dideacyl GM_3; D, N-trifluoroacetyl N'-acetyl dideacyl GM_3; E, lysoGM_3. The spectrum of GM_3 was measured without deuterium oxide.

NMR study

To confirm that each reaction proceeded properly, the reaction products from GM_3 were examined by proton NMR spectrometry as shown in **Fig. 2**. Chemical shifts of the partial protons in the compounds at respective reactions for the formation of lysoGM_3 and lysoGM_1 are presented in **Table 1**. N,N'-dideacylGM_3 possessed neither of the two amide protons on ceramide and N-glycolyl-neuraminic acid nor cisoid olefinic or α-methylenic protons on fatty acid as compared to the starting GM_3 containing N-glycolyl neuraminic acid (see Table 1), confirming the removal of the N-glycolyl group from the sialic acid and of the fatty acid from the ceramide moiety. The spectra of the N-trifluoroacetyl N'-deacyl-GM_3 and -GM_1, however, did not show the insertion of a trifluoroacetyl group, though the presence of the N-trifluoroacetyl group was clearly demonstrated by negative FAB-MS (see below). N-Acetylation of the N-trifluoroacetyl GM_3 derivative was confirmed by the presence of an amide proton at 7.860 ppm and an N-acetyl methyl group at 1.908 ppm in the product, which should be assigned to the sialic acid from their chemical shifts. In the mild saponification product (lysoGM_3) of the N-trifluoroacetyl N'-acetyl derivative, there remained an amide proton at 7.778 ppm and N-acetyl methyl protons at 1.904 ppm. The amide proton was assigned to that on sialic acid from the chemical shift referred to in a previous NMR study of glycosphingolipids (10). Furthermore, the spectra of the final product and starting N,N'-dideacyl GM_3 were identical to those of these lipids reported previously (4, 5). As summarized in Table 1, the spectrum of the N-trifluoroacetyl N'-acetyl derivative which was obtained from N,N'-dideacyl GM_1 by N-trifluoroacetylation followed by N-acetylation demonstrated the absence of a fatty acyl group and the

TABLE 1. Chemical shifts (ppm) in proton NMR of the synthetic intermediates for lysogangliosides

	H-1				Sph		N-Ac		Amide		
Derivative	I	II	III	IV	H-4	H-5	GalNAc	NeuAc	Cer	GalNAc	NeuAc
GM_3[a]	4.179	4.242			5.405	5.573		1.891	7.176		7.828
NeuLacSph[b]	4.206	4.252			5.482	5.618					
NeuLacSphTFAc[c]	4.228	4.257			5.388	5.626					
NeuAcLacSphTFAc[d]	4.260	4.275			5.487	5.750		1.908			7.860
NeuAcLacSph[e]	4.228	4.249			5.389	5.626		1.904			7.778
GM_1[a]	4.174	4.296	4.883	4.275	5.394	5.570	1.801	1.902	7.139	7.306	7.833
II^3NeuGg_4Sph	4.201	4.272	4.894	4.257	5.458	5.632	1.786			7.333	
$II^3NeuGg_4SphTFAc$	4.205	4.271	4.876	4.251	5.368	5.604	1.783			7.289	
$II^3NeuAcGg_4SphTFAc$	4.204	4.290	4.891	4.275	5.367	5.607	1.790	1.882		7.306	7.748
$II^3NeuAcGg_4Sph$	4.228	4.288	4.892	4.268	5.456	5.684	1.788	1.877		7.367	7.648

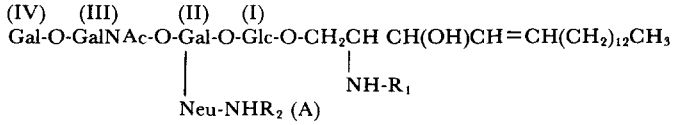
[a]Measured in the absence of D_2O.
[b]Demonstrated N,N'-dideacyl GM_3.
[c]Demonstrated N-trifluoroacetyl N'-deacetyl dideacyl GM_3.
[d]Demonstrated N-trifluoroacetyl N'-acetyl dideacyl GM_3.
[e]Demonstrated lysoGM$_3$.

presence of two N-acetyl methyl protons at 1.882 and 1.790 ppm, which were assigned to the N-acetyl neuraminic acid and N-acetyl galactosamine moieties, respectively. After removal of the N-trifluoroacetyl residue from the GM_1 derivative, the spectrum of the product was identical to that of lysoGM$_1$ measured previously (5).

FAB-MS study

The molecular mass of the synthetic intermediates in the preparation of lysogangliosides was analyzed by negative FAB-MS spectra. Fragment ions of the ganglioside derivatives were summarized in **Table 2**. The pseudomolecular negative ion *m/z* 871 due to [M-H] of dideacylated GM_3 (M_r = 872) was observed together with [M-Neu], *m/z* 622, confirming the remainder of a neuraminic acid moiety at the nonreducing terminus after the saponification reaction. The intensities of the *m/z* 460, [M-Neu-Gal] and *m/z* 298, [M-Neu-Gal-Glc] were weak, probably due to an unsuitable matrix used for the stability of the fragments. On the other hand, N-trifluoroacetyl N'-deacetyl GM_3 clearly gave ions *m/z* 967 due to [M-H], *m/z* 718 [M-Neu], *m/z* 556 [M-Neu-Gal], and *m/z* 394 [M-Neu-Gal-Glc]. The observation of ion *m/z* 718 [M-Neu] but not *m/z* 622 [M-(N-trifluoroacetyl Neu)] indicates that trifluoroacetylation of dideacylated GM_3 occurred exclusively at the amino residue on the sphingosine. N-Trifluoroacetyl N'-acetyl GM_3 demonstrated ion *m/z* 1009 due to [M-H] and the same other fragment ions as the N-trifluoroacetyl N'-deacetyl GM_3. The final product gave ions *m/z* 913 due to [M-H] of

TABLE 2. Fragment ions (*m/z*) observed by negative FAB/MS of the synthetic intermediates for lysogangliosides



Derivatives			Fragment Ions (*m/z*)							
GM_3 (I, II, A)	R_1	R_2	M-H	M-A	M-A, II	M-A, II, I				
NeuLacSph	H	H	871	622	(460)[a]	(298)				
NeuLacSphTFAc	TFAc	H	967	718	556	394				
NeuAcLacSphTFAc	TFAc	Ac	1009	718	556	394				
NeuAcLacSph	H	Ac	913	622	(460)	(298)				
GM_1 (I, II, III, IV, A)			M-H	M-A	M-IV	M-IV, III	M-A, IV	M-A, IV, III	M-A, IV~II	M-A, IV~I
II^3NeuGg_4Sph	H	H	1236	(987)	1074	(871)	(825)	(622)	460	(298)
$II^3NeuGg_4SphTFAc$	TFAc	H	1332	1083	1170	967	921	718	556	394
$II^3NeuAcGg_4SphTFAc$	TFAc	Ac	1374	1083	1212	1009	921	718	556	394
$II^3NeuAcGg_4Sph$	H	Ac	1278	(987)	(1116)	(913)	(825)	(622)	(460)	(298)

[a]Relative intensity of the *m/z* value in parentheses was low.

lysoGM$_3$ and *m/z* 622, [M–Neu]. Structural information similar to that of lysoGM$_3$ was obtained from the spectra of the synthetic intermediates for lysoGM$_1$. The N-deacylated product of GM$_1$ gave *m/z* 1236 and *m/z* 1074 due to ions [M–H] and [M–Gal], respectively, indicating the presence of an N-acetyl group from the N-acetylgalactosamine after the saponification reaction. The N-trifluoroacetyl N'-deacetyl derivative of dideacylGM$_1$ showed *m/z* 1332, 1083, 1170, 967, and 921 due to ions [M–H], [M–Neu], [M–Gal], [M–Gal–GalNAc], and [M–Neu–Gal], respectively, in addition to the fragment ions observed in N-trifluoroacetyl N'-deacetyl dideacyl GM$_3$. The N-trifluoroacetyl N'-acetyl derivative of dideacyl GM$_1$ afforded ions *m/z* 1374 [M–H], *m/z* 1212 [M–Gal], *m/z* 1009 [M–Neu–Gal], and the other same fragments as N-trifluoroacetyl lyosGM$_3$. These fragments indicate the occurrence of N-trifluoroacetylation of sphingosine and the following N-acetylation at the neuraminic acid of dideacyl GM$_1$, similar to the reaction of dideacyl GM$_3$. Removal of the trifluoroacetyl residue from the N-trifluoroacetyl N'-acetyl derivative of dideacyl GM$_1$ gave a product having ions *m/z* 1278 [M–H], *m/z* 1116 [M–Gal], and *m/z* 913 [M–Gal–GalNAc], which was identical to that of lysoGM$_1$ reported previously (5).

DISCUSSION

Preparation of lysogangliosides was first reported by Sonnino et al. (2) using direct saponification of parent gangliosides. The selective N-defatty acylation of the gangliosides by the saponification, however, resulted in a low yield of the lysoganglioside. Neuenhofer et al. (5) and Nores et al. (4) independently improved the preparation method for the lysoganglioside using corresponding dideacylated gangliosides. The former group used Fmoc-Cl as a selective blocking agent of sphingosyl amino residue under a bilayer system composed of aqueous alkaline and ether, followed by N-acetylation, whereas the latter directly N-acetylated the liposome containing dideacylated ganglioside and other lipids. Though many steps were required to obtain the lysogangliosides, the yields through the latter two methods were much better than that from direct saponification. However, since the reactivity of Fmoc-Cl toward the amino residue was relatively higher under basic conditions, the reaction with dideacylated ganglioside afforded several by-products, causing a low yield of lysoganglioside. For example, the reaction of dideacylated GM$_3$ with Fmoc-Cl gave N-deacyl N'-Fmoc dideacyl GM$_3$ (NeuFmoc-LacSph) and N,N'-di-Fmoc dideacyl GM$_3$ (NeuFmoc-LacSphFmoc) as minor products (totally more than 30%) in addition to the major N-Fmoc N'-deacetyl dideacyl GM$_3$ (NeuLac-SphFmoc), which was an intermediate for the desired product. Following N-acetylation and mild hydrolysis of NeuFmoc-LacSph and NeuFmoc-LacSphFmoc, these derivatives were converted to N-acetyl N'-deacetyl dideacyl GM$_3$ (NeuLac-AcSph) and starting dideacyl GM$_3$, respectively.

In this report, selective N-trifluoroacetylation at the sphingosyl amino group of dideacylated ganglioside was performed under a bilayer system, giving a higher yield of lysoganglioside. The reaction of ETFTA with dideacylated gangliosides was slower and the reactivity was lower than that of Fmoc-Cl, resulting in low production of by-products. Since both blocking reagents are soluble in an organic solvent rather than water, the blocking reactions may have predominantly occurred in the organic layer, in which the lipid moiety of dideacylated gangliosides tended to localize. Furthermore, the trifluoroacetyl residue bound on the amino group was completely removed in a mild alkaline solution at pH 11 (6), in which the N-acetyl group was not cleaved.

In the proton NMR study, amide protons on neuraminic acid and ceramide moieties, olefinic and α-methylene protons on the fatty acid moiety, and N-acetylmethyl protons informed the chemical structures of the intermediates and the final products. However, the NMR spectrum was unsuitable for the structural confirmation of the N-trifluoroacetyl derivatives, though the negative FAB–MS technique contributed greatly to identifying the structure. Though the reason is not known, the trifluoroacetyl derivatives fragmented well and the fragments were stabilized in the glycerol-matrix in the negative FAB–MS.

Lysogangliosides could be immobilized at their lipid moiety on adsorbent containing activated carboxilic acid to make an affinity column. The lysogangliosides may also be developed as a probe of affinity labeling toward proteins with a ganglioside metabolism after adequate chemical modification of the structure, other than being employed as modulators of transmembrane signaling.

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